



High Throughput Analysis of Stress Growth Response in *Shewanella oneidensis* MR-1

Natalie Katz, Terry C. Hazen, Rick Huang, Dominique Joyner, and Sharon E. Borglin
Virtual Institute for Microbial Stress and Survival, Lawrence Berkeley National Laboratory

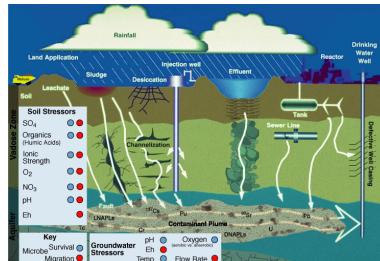


Abstract

Shewanella oneidensis MR-1 has shown extraordinary metabolic diversity through its use of a variety electron acceptors. It can grow both aerobically and anaerobically, and can use nitrate, fumarate, sulfur compounds, and oxidized metals as electron acceptors. To study the stress of *S. oneidensis* to nitrate, nitrite, and sodium chloride, this bacterium had to be made detectable to the Biolog Omnilog Phenotype Microarray™ system. The Omnilog machine uses digital imagery sensing technology to track changes in the turbidity of cultures growing in individual wells of a microarray plate over a given time period. Omnilog was calibrated with three other methods- OD₆₀₀ readings, OD₅₅₀ readings, and cell density-in order to determine the generation time of *S. oneidensis* by correlating the digital readings produced with known cell and optical density measurements. This calibration was necessary because in early stages of growth, *S. oneidensis* is opaque and scarcely visible in liquid media. Consequently, the Omnilog machine showed extraordinarily long lag phases of growth, coupled with short log phase and entrance into stationary phase extremely quickly. Omnilog also posted unimpressive maximum digital unit readings once stationary phase had been reached; maximum values usually did not exceed 100 units (maximum reading obtainable for other bacteria is 450). To resolve this problem, Dye Mix A (tetrazolium-based) from Biolog, Inc. was added to the liquid cultures of *S. oneidensis* at a final concentration of 0.5x. The maximum digital units posted were ≥ 250, staying within the linear range of Omnilog. Subsequent growth curves had much shorter lag phases, longer log phase growth and eventual passage into stationary phase, conveying a better representation of bacterial growth. Use of the dye almost virtually eliminated the variation in the digital units from one reading to the next. Future studies of the stress response of *S. oneidensis* to nitrate, nitrite and sodium chloride will be conducted in media containing 0.5x Dye Mix A.

Background

Since the 1990s, the US Department of Energy (DOE) has focused on decontaminating and remediating soil and water surrounding facilities where nuclear research, production, and testing had been conducted by DOE and its predecessor agencies. Based on current remediation technology, DOE estimates that it will take more than 70 years and could cost over \$300 billion to decontaminate these nuclear production facilities and sites.



Bioremediation has shown to be a cost-effective way of eliminating and/or containing these hazardous compounds through the use of microorganisms to reduce, eliminate, contain, or transform the contaminants to non-hazardous or less hazardous forms. However, before these hazardous sites can be effectively decontaminated, further research must be done into the biological, chemical, and physical factors that influence the subsurface mobilization and immobilization of metals and radionuclides.

Shewanella oneidensis is one the principle research focuses for DOE on bioremediation metals and radionuclides, due to it's ability to actively reduce a large number of metals under both aerobic and anaerobic conditions. Our group has been developing high throughput screening techniques for growth and stress conditions and phenotypic characterization. Biolog, Inc., whom manufactures the Omnilog™ machine, engineered a tetrazolium based dye mix that could be included in bacterial cultures to measure their growth. It was hypothesized that this dye mix, which turns to a deep purple formazan precipitate upon reduction, could make *S. oneidensis* more visible to the digital camera in Omnilog™ system, thus alleviating the problems associated with low density units recorded and inconsistency in generation times amongst controls. We have used this system to successfully estimate minimum inhibitory concentrations (MIC) of various stressors in a matter of hours, as opposed to weeks required using more conventional techniques.

- 10% inoculum of primary culture
- Defined minimal media
- Nitrate, Nitrite, Salt (Sigma-Aldrich Chemicals)
- Dye Mix A, Biolog Inc., Hayward, CA
- Biolog Omnilog Phenotype Microarray System
- 96-well microarray plates
- 6 duplicates run for each condition examined
- Controlled incubation temperature
- Automated sampling and logging at 15 min intervals for more than 200 h
- 50 plates handled at one time

Materials and Methods



Results

These data demonstrate the growth characteristics of *Shewanella oneidensis* MR-1 with a final concentration of 0.5x dye included in the growth medium. Inclusion of the dye resulted in shorter lag phases, longer log phases, and a gradual entrance into stationary phase.

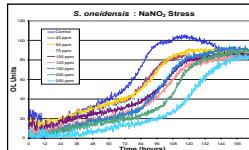


Figure 1. Example of growth of *S. oneidensis* in response to nitrate stress, without dye mix present in the growth medium. The length of the lag phase ranges from 70-100 hours; log phase growth ranges from 15-20 hours in length. The maximum density detected by the Omnilog did not exceed 110 units.

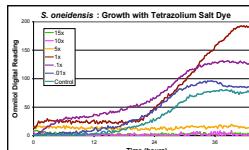


Figure 2. Five concentrations of dye mix were examined in order to determine the best concentration to use with actively growing cells. A final concentration of 0.5x dye in the medium was chosen based on these results.

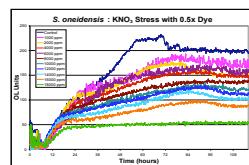


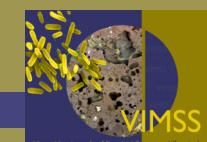
Figure 4. Example of growth of *S. oneidensis* in response to nitrate stress, with potassium as the carrier salt. Lag phase growth was less than 12 hours, and log phase growth ranged from 10-80 hours in length. The maximum density detected by the Omnilog was almost 250 units.

MIC's & Generation Times

The MIC is defined as the concentration at which a ≥ 50% inhibition of final cell yield is seen.

	MR1 Medium		LS4D Medium	
Stressor	MIC	Gen Time (hours)	MIC	Gen Time (hours)
Control	N/A	2.05	N/A	2.22
NaCl	100 mM	2.16	300 mM	3.21
KCl	250 mM	3.69	250 mM	3.04
NaNO3	250 ppm	3.22	1000 ppm	2.25
KNO3	6000 ppm	4.54	1500 ppm	2.97
NaNO2	125 ppm	4.21	125 ppm	2.74
NaO2	125 ppm	3.44	125 ppm	6.41

Table 2. Minimum Inhibitory Concentrations (MIC's), and their associated calculated generation times as determined with 0.5x tetrazolium-based dye mix in the medium.



S. oneidensis : NaNO ₃ Stress					
Concentration (mM)	Gen. Time (hours)	Concentration (mM)	Gen. Time (hours)	Concentration (mM)	Gen. Time (hours)
0 mM	23.5	0 ppm	32.2	0 ppm	42.2
50	28	1500	41.8	29	48.4
100	32.8	2500	41.8	79	53.1
150	45	4000	42.2	100	40.6
200	68.6	4500	102.8	125	44.5
250	81.7	5500	152.1	125	44.5
300	140.2	6500	199.8	150	45.5
350	44	7500	146.8	200	46.2
400	52	10000	125.0	250	44.2
450	117.4				

Table 1. Generation times calculated in response to salt, nitrate and nitrite stress. The generation times of the controls were not consistent, and the other calculated generation times ranged from 28 to 200 h per generation.

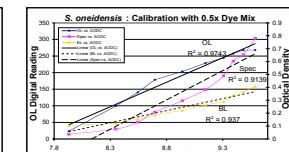


Figure 3. A calibration was done between the OMNILOG and direct cell counts for *S. oneidensis* grown in defined minimal medium with a final concentration of 0.5x dye included.

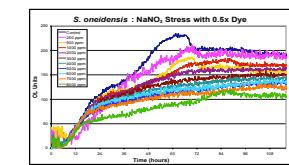


Figure 5. Example of growth of *S. oneidensis* in response to nitrate stress, with sodium as the carrier salt. Lag phase growth was less than 12 hours, and log phase growth ranged from 10-80 hours in length. The maximum density detected by the Omnilog was almost 250 units.

LS4D vs.. MR1 Defined Minimal Media

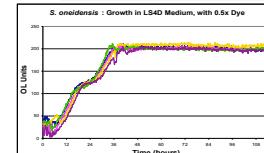


Figure 6. Control growth curves in LS4D medium from each of the stress response experiments. Growth with the dye mix was reproducible.

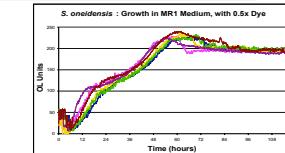


Figure 7. Control growth curves in MR1 medium from each of the stress response experiments. Growth with the dye mix was reproducible.

VIMSS, Flow Chart

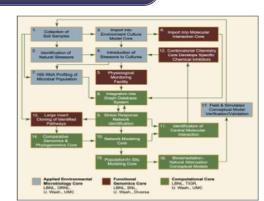


Figure 8. The Virtual Institute for Microbial Stress and Survival (VIMSS) pipeline seeks to identify stress response pathways induced by various environmental factors by combining multiple simultaneous analyses, such as proteomics, metabolomics, lipidomics, transcriptomics, and phenomics, in an effort to conceptualize these pathways.

Conclusions and Future Work

- The optimal concentration of dye mix used in the cultures was determined to be 0.5x because it stayed within the linear range of 50-300 Omnilog units and was not toxic to microbial metabolism.
- Growth with the dye, when compared to growth without it, resulted in shorter lag phases, longer log phases, and a gradual increase in cell densities towards stationary phase; inclusion of the dye resulted in a maximum density of 250 Omnilog units- a full order of magnitude higher than densities recorded without dye mix included in the medium.
- A comparison was done between sodium and potassium as the carrier ions for nitrate, nitrite and salt stress in both MR1 and LS4D media. Preliminary results suggest that the sodium ion places additional stress on *S. oneidensis* when examining nitrate, nitrite or salt stress.
- The Omnilog is a rapid, high-throughput machine capable of determining multiple growth curves (up to 480) at once, in a couple of days; without the Omnilog these stress response studies would have taken several months, or longer, to complete.

These data suggest that use of a tetrazolium-based dye, when included in actively growing bacteria cultures, can make them more detectable to the Biolog Omnilog Phenotype Microarray™ system without interfering with normal microbial growth and metabolism. The Omnilog provided a rapid, reproducible, high-throughput method of determining growth curves in response to stress, and automatically recorded and saved the data. The information gathered from these stress response studies will be used to understand how these stressors affect the ability of the bacteria to reduce heavy metals and radionuclides at DOE contaminated sites. All information obtained from these analyses will then be transformed into a meaningful model of cellular regulatory networks used to counteract stress.

Acknowledgements

This work was part of the Virtual Institute for Microbial Stress and Survival supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomics Program; GTL through contract DE-AC03-76SF00098 between Lawrence Berkeley National Laboratory and the U.S. Department of Energy.

References

- Arkin, A., T. C. Hazen. 2002. Rapid deduction of stress response pathways in metalradionuclide reducing bacteria. http://vims.sciences.duke.edu/vims_proposal.html
- BioMetals of Metals and Radionuclides... What it is and How it Works. 2003. Natural and Accelerated Bioremediation Primer. 2nd Ed.
- Bochner, B. and M. Savageau. 1997. Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. Appl. Environ. Microbiol. 63:134-144.
- Middleton, S. S., R. B. Latmani, M. R. Mackey, M. H. Ellisman, B. M. Tebo, and C. S. Criddle. 2003. Metabolism of Cr(VI) by *Shewanella oneidensis* MR-1 produces cell-associated reduced chromium and inhibits growth. Biotechnol. Bioeng. 83:627-636.